

# Cr2, a Candidate Gene in the Murine *Sle1c* Lupus Susceptibility Locus, Encodes a Dysfunctional Protein

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## Summary

The major murine systemic lupus erythematosus (SLE) susceptibility locus, *Sle1*, corresponds to three loci independently affecting loss of tolerance to chromatin in the NZM2410 mouse. The congenic interval corresponding to *Sle1c* contains *Cr2*, which encodes complement receptors 1 and 2 (CR1/CR2, CD35/CD21). NZM2410/NZW *Cr2* exhibits a single nucleotide polymorphism that introduces a novel glycosylation site, resulting in higher molecular weight proteins. This polymorphism, located in the C3d binding domain, reduces ligand binding and receptor-mediated cell signaling. Molecular modeling based on the recently solved CR2 structure in complex with C3d reveals that this glycosylation interferes with receptor dimerization. These data demonstrate a functionally significant phenotype for the NZM2410 *Cr2* allele and strongly support its role as a lupus susceptibility gene.

## Introduction

Female (NZB × NZW)<sub>F1</sub> mice develop a clinical picture similar to human SLE, with the production of IgG anti-dsDNA autoantibodies and the evolution of a fatal immune complex-mediated glomerulonephritis. Disease susceptibility has been linked to genes derived from both the NZB and NZW parental strains. Various strategies have been used to identify the specific disease-related genes in these animals. One approach has utilized the lupus-prone NZM2410 strain, a recombinant inbred strain derived from (NZB × NZW)<sub>F1</sub> mice (Rudofsky et al., 1993). NZM2410 mice exhibit a severe lupus phenotype, including fatal glomerulonephritis. We have identified by linkage analysis three recessive loci strongly associated with lupus susceptibility in this strain (*Sle1*, *Sle2*, and *Sle3* located on chromosomes 1, 4, and 7, respectively) (Morel et al., 1994).

In order to identify specific disease-related genes, B6.NZM congenic mice containing the individual

NZM2410-derived disease susceptibility loci were generated on a C57BL/6 background (Morel et al., 1996). Functional analysis of these mice showed that each locus confers a unique phenotype. B6.*Sle1* mice produce high levels of anti-nuclear antibodies, indicating a loss of tolerance to chromatin (Mohan et al., 1998), while B6.*Sle2* demonstrate B cell hyperactivity (Mohan et al., 1997) and B6.*Sle3* exhibit dysregulation of the T cell compartment (Mohan et al., 1999). Although none of the individual congenic mice manifest full-blown lupus, severe disease can be reconstituted by combining *Sle1* with *Sle2* or *Sle3*, demonstrating that *Sle1* is critical for the development of lupus nephritis (Morel et al., 2000). In addition, we have shown the existence of negative modifier loci (*Sles1-4*) that, by specifically suppressing the *Sle1* phenotypes, completely inhibit SLE pathogenesis (Morel et al., 1999). The *Sle1* interval is located in a region of chromosome 1 that has been linked with SLE susceptibility in multiple human ethnic groups and mouse strains (Harley et al., 1998; Wakeland et al., 1999). Taken together, these data suggest that loss of tolerance to chromatin mediated by *Sle1* is essential for disease pathogenesis and that the proteins encoded by these loci and the pathways on which they impact may be important targets for disease intervention in SLE.

Recently, we have shown that *Sle1* corresponds to at least three loci (*Sle1a*, *Sle1b*, and *Sle1c*) that each independently affects the loss of tolerance to chromatin, but presents distinct immunological characteristics (Morel et al., 2001). In contrast to B6.*Sle1a* and B6.*Sle1b*, the B6.*Sle1c* congenic strain is characterized by antibodies to chromatin that are not restricted to the H2A/H2B/DNA subnucleosome and that are produced equally in males and females. In addition, the level of serum IgM in B6.*Sle1c* mice is lower than in B6 controls, and they accumulate a significantly higher percentage of activated CD4<sup>+</sup> splenic T cells (Morel et al., 2001). The congenic strain B6.*Sle1c* contains within its interval the gene *Cr2*, which encodes complement receptors 1 and 2 (CR1/CR2, CD35/CD21) by alternative splicing of a single mRNA transcript (Kurtz et al., 1990; Molina et al., 1990). In humans, CR1 and CR2 are generated from two distinct but closely linked genes on chromosome 1. The murine *Cr2* gene encodes both the 190 kDa CR1 protein consisting of 21 repeating structures (termed short consensus repeats or SCRs), a transmembrane domain, and a short cytoplasmic tail, as well as the alternatively spliced 140 kDa CR2 protein, containing the 15 membrane-proximal SCRs of CR1 plus its transmembrane domain and cytoplasmic tail. CR1 and CR2 are surface glycoproteins that, in the mouse, are located almost exclusively on mature B cells and follicular dendritic cells (FDC). These receptors bind C3 and C4 degradation products that have become covalently bound to antigen or immune complexes in the process of complement activation. Previous studies have demonstrated a role for CR2 in humans or CR1/CR2 in mice in lowering the threshold for B cell activation (Carter et al., 1988; Fingerth et al., 1989; Luxembourg and Cooper, 1994), rescuing B cells from surface IgM (sIgM)-mediated apo-

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ptosis (Kozono et al., 1995), targeting immune complexes to germinal centers (Klaus, 1978; Papamichail et al., 1975), and processing and presenting antigen (Arvieux et al., 1988; Boackle et al., 1997, 1998; Lanza-vecchia et al., 1988; Thornton et al., 1994). Mice rendered deficient in CR1/CR2 by homologous recombination (*Cr2*<sup>-/-</sup>) have defects in primary and secondary IgG responses to T-dependent antigens, germinal center formation, and the generation of memory B cells (Ahearn et al., 1996; Croix et al., 1996; Molina et al., 1996; Wu et al., 2000).

CR1 and CR2 have been proposed to play a role in the development of SLE. Patients with SLE have ~50% lower levels of these receptors on their B cells (Levy et al., 1992; Marquart et al., 1995; Wilson et al., 1986). MRL/lpr mice, which develop severe lupus-like disease, exhibit lower levels of these receptors on B cells prior to the onset of clinical disease, suggesting that the reduction in CR1/CR2 expression may be pathogenic (Takahashi et al., 1997). Furthermore, when the inactivated *Cr2* allele was introduced in a homozygous state into the B6/lpr background, the autoimmune disease that developed, which is usually mild and occurs late, was more severe and had a much earlier onset (Prodeus et al., 1998). These data suggest that alterations in expression or function of CR1/CR2 may affect negative selection of autoreactive B cells, resulting in the initiation or exacerbation of SLE.

Because of the previous association of CR1/CR2 deficiency with autoimmune disease and the location of the *Cr2* gene within the *Sle1c* disease susceptibility locus, we analyzed the *Sle1c* (NZW) allele of *Cr2* in B6.*Sle1c* mice for alterations in structure, function, and expression. We identified a structural difference in a critical ligand binding domain of *Sle1c* CR1/CR2, which results in significant impairment in receptor function. These results demonstrate a functionally significant effect of the NZM2410 *Cr2* allele and strongly support its role as a disease susceptibility gene in this interval.

## Results

### The NZW Allele of *Cr2*, Located within the *Sle1c* Disease Susceptibility Interval, Generates Protein Products that Are Structurally Different from Their B6 Counterparts

The subcongenic strain B6.*Sle1c* was derived from the parental B6.*Sle1* strain by generation of mice with recombinant intervals that maintained a positive phenotype for loss of tolerance to chromatin (Morel et al., 2001). B6.*Sle1c* mice specifically contain the congenic NZM2410 interval that spans the microsatellite marker D1Mit274 to the telomeric end of chromosome 1. We have previously shown that this NZM2410 region was derived from NZW (Morel et al., 1996). The *Sle1c* interval is approximately 3 cM in length, and contains several potential SLE susceptibility genes, including *Cr2*, *Crry*, and *Cd34* (Figure 1).

To determine whether the *Cr2* gene products were altered in B6.*Sle1c* mice, CR1 and CR2 were immunoprecipitated from surface-biotinylated splenic cell suspensions with a mAb to CR1/CR2, 7E9, which binds to an epitope within the SCRs common to both receptors

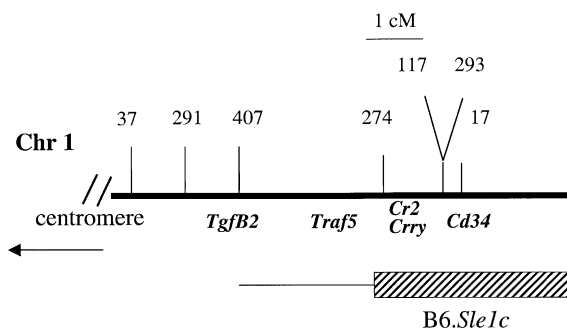


Figure 1. Genetic Map of the B6.*Sle1c* Congenic Strain

The upper part of the figure represents the telomeric end of chromosome 1, with D1MIT markers shown. The map location of these markers has been calculated on a panel of 493 (B6 × NZM2410) meioses, with D1MIT37 located 101 cM from the centromere as the anchor locus. Under the chromosome 1 line are shown the approximate positions of relevant genes in relation to these markers. The position of the NZM2410-derived congenic interval for B6.*Sle1c* is shown as a hatched box, with the thin line between D1MIT407 and D1MIT274 indicating the area of recombination between the NZM2410 and B6 genomes.

(Molina et al., 1994). After SDS-PAGE and immunoblotting with HRP-streptavidin, CR1 and CR2 from B6.*Sle1c* were found to migrate at a higher molecular weight than the corresponding proteins from B6 animals (Figure 2A). This difference was due to differential glycosylation of the proteins, as treatment with PNGaseF reduced the proteins from the two strains to equivalent molecular weights (Figure 2B). To ensure that this difference did not reflect a global alteration in glycosylation of B cell surface receptors in the B6.*Sle1c* strain, CD19 and CD22 were analyzed in a similar fashion and were found to be similar in size in B6 and B6.*Sle1c*, whether glycosylated or deglycosylated (Figure 2B). These data demonstrate that the enhanced glycosylation of CR1 and CR2 in B6.*Sle1c* is an alteration unique to these two proteins.

### A Single-Nucleotide Polymorphism (SNP) in NZW *Cr2* Introduces a Novel N-Linked Glycosylation Site in the Ligand Binding Domain of CR1/CR2

Having identified an alteration in the structure of CR1/CR2, the sequence of *Cr2* in B6.*Sle1c* mice was then determined in order to identify nucleotide polymorphisms that might confer new N-linked glycosylation sites. At nucleotide 1342, a base change of C to A was identified, resulting in an amino acid change of histidine to asparagine. This introduced a new N-linked glycosylation site into SCR1 of CR2 (Figure 2C), which corresponds to SCR7 of CR1. It also resulted in a new *BsmI* restriction endonuclease site, allowing genomic DNA from a panel of autoimmune and nonautoimmune mouse strains to be tested for the presence of this polymorphism. In addition to NZW, only two other strains in this group share this polymorphism: NOD, which develops autoimmune diabetes (Leiter, 1993), and SWR, which when crossed with NZB results in female progeny that develop a severe lupus-like disease (Eastcott et al., 1983). Both of these strains share common ancestry through their derivation from "Swiss" mice (Leiter, 1998). Four other autoimmune strains (BXSB, MRL, NZB, and

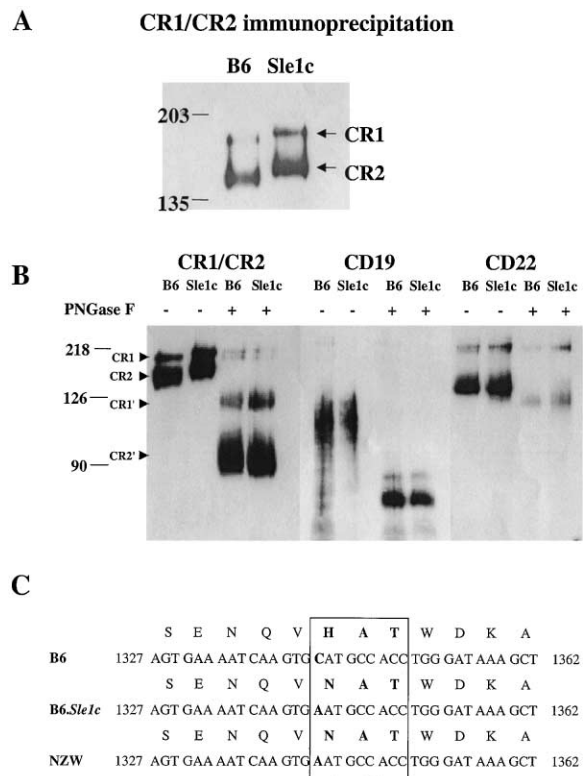


Figure 2. Increase in Molecular Weight of CR1/CR2 in B6.Sle1c B Lymphocytes due to Differential Glycosylation of the Proteins

(A) Splenic cell suspensions were depleted of RBC and surface biotinylated. CR1/CR2 were immunoprecipitated with mAb 7E9, which binds an epitope shared by the two receptors. Immunoprecipitates were analyzed by SDS-PAGE, transferred to a nitrocellulose filter, and detected by HRP-streptavidin.

(B) Immunoprecipitates of CR1/CR2, CD19 and CD22 from surface biotinylated splenic cells were either untreated (-) or treated (+) with PNGaseF prior to SDS-PAGE and HRP-streptavidin Western blotting.

(C) Nucleotide and amino acid sequences demonstrating the single-nucleotide polymorphism and amino acid change in SCR1 of CR2 (SCR7 of CR1) that introduces a novel N-linked glycosylation site. Sequences shown were generated by RT-PCR of splenic mRNA. The polymorphic nucleotide was confirmed by restriction digestion of independent RT-PCR products using *BsmI*.

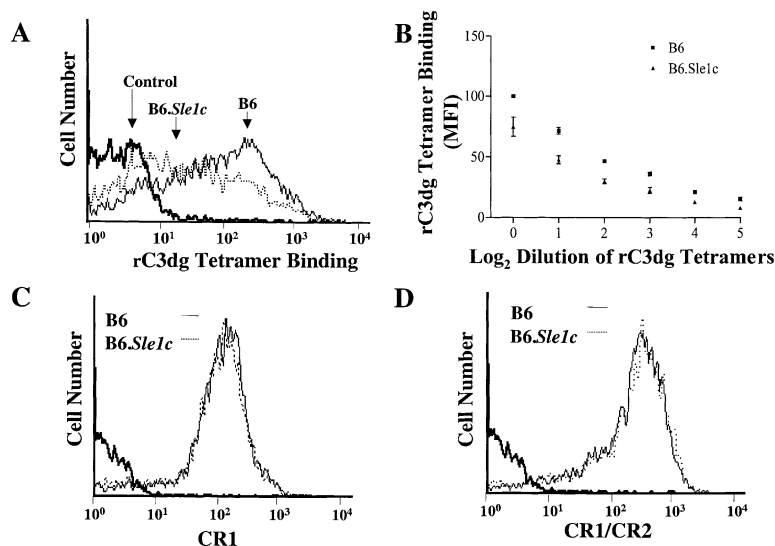
SJL) as well as five nonautoimmune strains (BALB/c, DBA/2, AKR, C3H, and FVB) were all negative by RFLP for this 1342 C→A polymorphism (Table 1). Interestingly, CR1/CR2 in SWR and NOD also migrate at higher apparent molecular weights than the B6 proteins in SDS-PAGE, with initial analyses of the glycosylation status of these proteins strongly suggesting that the molecular weights of the core proteins are equivalent (data not shown). No other glycosylation sites introduced by unique polymorphisms in the N2W, SWR and NOD *Cr2* genes were identified. These data suggest that the novel glycosylation site introduced by the polymorphism in SCR1 of CR2 is utilized in each strain in which it is present.

A total of 16 nucleotide sequence differences were identified in the NZW *Cr2* allele as compared with B6. This represents a mutation rate that is ~3-fold higher than expected, based on genotyping of SNPs between

Table 1. Analysis of B6 and B6.Sle1c Polymorphisms in Autoimmune and Nonautoimmune Strains

nt Polymorphisms	aa Changes	Location	RFLP	B6	B6.Sle1c	NZW	NOD	SWR	BXSB	MRL	NZB	SJL	BALB/c	DBA/2	AKR, C3H, FVB
(A→G) 409	Met → Val	SCR2	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
+AGG (873-874)	+Lys	SCR5	-FokI	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(C→A) 1342	His → Asn	SCR7/SCR1	+BsmI	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(C→T) 1700	Thr → Ile	SCR9/SCR3	-BsrI	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(C→G) 1885	Pro → Ala	SCR10/SCR4	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(A→C) 2518	Thr → Pro	SCR13/SCR7	+MnlI	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(G→A) 2690	Arg → His	SCR14/SCR8	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(G→A) 3025	Asp → Asn	SCR16/SCR10	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(A→C) 3296	Lys → Thr	SCR18/SCR12	+MunI	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(G→A) 3722	Ser → Asn	SCR20/SCR14	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(G→T) 4108	Gly → Cys	TM	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND
(A→C) 4138	Ser → Arg	CYTO	-	-	+	+	-	-	-	ND	ND	ND	ND	ND	ND

Polymorphisms identified in the sequence of B6.Sle1c *Cr2* and comparisons with other strains. Resulting amino acid changes are in the second column and their location in the linear SCR structure of the CR1 and CR2 proteins, respectively, are indicated in the third column. Also, the introduction or deletion of restriction sites by the polymorphism is shown in column 4, and the presence (+) or absence (-) of the polymorphisms in various autoimmune and nonautoimmune strains is demonstrated in the remainder of the table, as identified by RFLP of PCR products from genomic DNA or direct sequencing of RT-PCR products from splenic mRNA. ND = not determined.



**Figure 3. Impaired Ability of B6.Sle1c CR1/CR2 to Bind rC3dg Tetramers**

(A) rC3dg tetramers prepared from 2  $\mu$ g of biotin-rC3dg and 0.4  $\mu$ g of PE-streptavidin were incubated with RBC-depleted splenic cells for 30 min at 4°C. FITC B220-positive B6.Sle1c cells (dashed line) and B6 cells (thin solid line) were analyzed for binding of immune complexes by flow cytometry. Binding of PE-SA prepared without the addition of rC3dg to B220<sup>+</sup> B6 cells is demonstrated by the thick line. Identical control binding to B220<sup>+</sup> B6.Sle1c cells was seen (data not shown). These data are representative of three separate experiments.

(B) A stock mixture of 2  $\mu$ g b-rC3dg and 0.4  $\mu$ g of PE-streptavidin was prepared. B6 and B6.Sle1c B cells were incubated with dilutions of rC3dg tetramers then analyzed by flow cytometry. In each of three separate experiments, the sample with the maximal mean fluorescence intensity was set at a relative value of 100 and the other samples normal-

ized to this value. The average and SEM of the normalized values for mean fluorescent intensity (MFI) are demonstrated.

(C and D) RBC-depleted splenic cells were stained with anti-CR1 (8C12; C) and anti-CR1/CR2 (4E3; D) and B220-positive B6.Sle1c cells (dashed line) and control B6 cells (thin line) were analyzed for expression of these receptors by flow cytometry. Binding of an isotype control antibody (thick line) is also indicated. These data are representative of three separate experiments.

lab strains (Lindblad-Toh et al., 2000). These sequence differences included 11 SNPs that resulted in amino acid changes and one 3 base insertion, resulting in the addition of a lysine residue in SCR5 of the CR1 protein (Table 1). In addition, four SNPs resulted in no amino acid changes (A  $\rightarrow$  G at nucleotide 243, T  $\rightarrow$  C at nucleotide 933, T  $\rightarrow$  G at nucleotide 2367, and C  $\rightarrow$  T at nucleotide 2736). Two other single nucleotide changes (C  $\rightarrow$  G at nucleotide 908, G  $\rightarrow$  C at nucleotide 909) and one 6 base deletion of nucleotides 4038–4043, although different from the published sequence for *Cr2* (Fingerroth, 1990; Kurtz et al., 1990; Molina et al., 1990), were identified in both B6 and B6.Sle1c.

In addition to the polymorphism in SCR7/SCR1 noted above, six additional alterations were tested in other strains using RFLP-PCR or direct sequencing (Table 1). The polymorphisms in SCR13/SCR7 and SCR16/SCR10 had the same strain distribution pattern as the SCR7/SCR1 polymorphism, while the SCR5 polymorphism was shared with SJL, and the SCR9/SCR3 polymorphism was shared with MRL, NZB, BALB/c, and DBA/2. The SNPs in the transmembrane and cytoplasmic domains were unique to NZW.

#### Binding of C3dg Ligand to B6.Sle1c B Cells Is Decreased

SCR1 and SCR2 of CR2 (SCR7 and SCR8 of CR1) serve as the primary ligand binding site for C3d- and C3dg-degradation products of complement covalently bound to antigen (Molina et al., 1994; Pramoonjago et al., 1993). We reasoned that the introduction of a new N-linked glycosylation site in SCR1 of CR2 in B6.Sle1c could interfere with ligand binding. To test this hypothesis, recombinant C3dg tetramers (rC3dg tetramers), prepared with biotinylated recombinant human C3dg (b-rC3dg) and PE-streptavidin, were bound to B6 and B6.Sle1c splenic cell preparations and analyzed by flow cytometry. Previous studies have shown that the  $K_d$  of

human C3d for mouse CR2 is indistinguishable from that for human CR2 (Molina et al., 1991). In addition, a human CR2 transgene can reconstitute the immunological defects identified in *Cr2*<sup>-/-</sup> mice (Marchbank et al., 2000). Based on these data, we believe that human rC3dg is an acceptable reagent for the study of endogenous CR2 function in mice.

Most of the B6.Sle1c B cells bound tetramers at levels more than an order of magnitude lower than B6 (Figure 3A). The mean fluorescence intensity (MFI) of tetramer binding to B6.Sle1c B cells was 25%–33% lower than to B6, with this difference being maintained over each dilution of the tetramers (Figure 3B). To ensure that this decrease in ligand binding was not due to altered expression of CR1/CR2 on B6.Sle1c B cells, analysis of CR1/CR2 mAb binding to B220<sup>+</sup> splenic cells was performed. 8C12 is specific for an epitope within SCR3 and SCR4 of CR1 (Molina et al., 1994), while 4E3 binds both receptors at a shared epitope within SCR1 and SCR2 of CR2 (SCR7 and SCR8 of CR1). The expression of CR1 and CR2 on the cell surface was equivalent in B6 and B6.Sle1c (Figures 3C and 3D). These results are also consistent with the similar levels of protein that are immunoprecipitated from each strain (Figure 2A and 2B). These results strongly suggest that decreased binding of ligand by the NZW *Cr2* allele is a result of the structural alteration identified in SCR7/SCR1 of CR1/CR2.

Binding of rC3dg tetramers to CR1 and CR2 is dependent upon the level of expression of the receptors, based on studies in K562 cells transfected with mouse CR1 and CR2 (data not shown). The overall binding of tetramers to B cells from each strain is heterogeneous, likely due to tetramer binding to splenic B cell subpopulations that express variable levels of CR2. CR1/CR2 expression varies among mouse splenic subpopulations by approximately 10-fold (Takahashi et al., 1997). We confirmed that the major B cell subpopulations (follicular and marginal zone) in B6 and B6.Sle1c mice are present in identi-

cal proportions (data not shown). Therefore, the variation in rC3dg tetramer binding identified within each strain is likely due to differences in receptor level on various subpopulations of B cells, while the differences in tetramer binding between the two strains are due to differences in receptor affinity or avidity.

#### CR1/CR2-Mediated Signaling Is Diminished in B6.*Slc1c* B Cells

As one means to determine whether the polymorphisms in B6.*Slc1c* Cr2 confer a functional effect, signaling through CR1/CR2 was studied. Cocrosslinking CR2 and sIgM has been shown to lower the threshold by which B cells can be activated through sIgM (Carter et al., 1988). We have previously demonstrated that calcium flux can be induced in resting murine B cells by cocrosslinking CR1/CR2 and sIgM with streptavidin-linked complexes of b-rC3dg and biotinylated anti-sIgM (b-7-6). At the subthreshold doses of b-rC3dg and biotinylated anti-sIgM used, a response could only be seen if both reagents were combined in the tetramers (Henson et al., 2001). Calcium flux induced by these complexes requires CR1/CR2, as it did not occur when stimulating *Cr2*<sup>-/-</sup> B cells. Crosslinking the receptors is also required, as no response was seen when the cells were incubated with b-rC3dg and biotinylated anti-sIgM in the absence of streptavidin.

When B6.*Slc1c* B cells were stimulated with tetramers prepared using subthreshold doses of b-C3dg and biotinylated anti-sIgM, several differences in calcium responses were identified in comparison with the responses from B6 B cells. The mean number of cells responding was decreased by 20%–40% (Figures 4A and 4C), and the mean amplitude of the response was decreased by 25%–50% (Figures 4B and 4D). Furthermore, the time to peak response was increased. When either a strong [F(ab)'<sub>2</sub> goat anti-mouse Ig; Figures 4E and 4F] or intermediate (tetramers containing higher doses of biotinylated anti-sIgM; data not shown) anti-sIgM stimulus was used to crosslink the B cell receptor (BCR) alone, B cells from both strains responded similarly in the numbers of cells responding and the amplitude of the response. These results demonstrate that B cells from the two strains generate equivalent signals through the BCR but that the altered B6.*Slc1c* CR2 allele is unable to function optimally to lower the threshold for B cell activation through sIgM.

#### Humoral Immune Response to T-Dependent Antigens Is Diminished in B6.*Slc1c* Mice

To further test the function of B6.*Slc1c* CR1/CR2, we analyzed B6.*Slc1c* mice for the presence of alterations in immune responses that have been identified in *Cr2*<sup>-/-</sup> mice. Several independent studies have shown that *Cr2*<sup>-/-</sup> and *Cr2*<sup>+/-</sup> mice have an impaired ability to mount normal humoral responses to T-dependent foreign antigens (Ahearn et al., 1996; Croix et al., 1996; Molina et al., 1996). B6 and B6.*Slc1c* mice were immunized intraperitoneally with the T-dependent antigen DNP-KLH in alum on days 0 and 21. Sera obtained on days 21 and 28 were tested by ELISA for the presence of IgM and IgG anti-DNP antibodies. There was a trend toward a significant decrease in both IgM and IgG anti-DNP levels

at 21 days in B6.*Slc1c* compared with B6 mice ( $p = 0.07$  for IgM and  $p = 0.10$  for IgG by Student's *t* test) (Figures 4G and 4H). At 28 days, levels of IgG anti-DNP were significantly decreased by ~20% in B6.*Slc1c* compared with B6 mice ( $p = 0.009$ ) (Figure 4H). Interestingly, this difference was primarily due to differences in IgG3 anti-DNP levels (O.D. at 1:100 dilution of  $997 \pm 290$  for B6 versus  $230 \pm 15$  for B6.*Slc1c*;  $p = 0.01$ ), which is the isotype predominantly affected in secondary responses to T-dependent antigens in *Cr2*<sup>-/-</sup> mice (Molina et al., 1996). These data provide additional support for an *in vivo* functional alteration of CR1/CR2 in B6.*Slc1c* mice.

#### Analysis of the Ligand Binding Domain of B6.*Slc1c* Cr2 by Molecular Modeling Based on the Human CR2 Crystal Structure

Recently, the 3-D structure of the complex containing the SCR1 and SCR2 ligand binding domain of human CR2 in association with C3d has been determined (Szakonyi et al., 2001). In the crystal structure, we found that CR2 forms dimers via SCR1-SCR1 interactions (Szakonyi et al., 2001) (Figure 5A). The dimer structure shows a moderate set of hydrogen bonds formed between residues of the E1  $\beta$  strand of one molecule and the D2  $\beta$  strand of another molecule, establishing a symmetrical contact with the E1 strands from each molecule running antiparallel to the other (Figure 5B). Of interest, molecular modeling of the mouse CR2 sequence reveals that the altered residue (asparagine) in the polymorphic SCR1 of B6.*Slc1c* CR2 is located at the equivalent position of human D53, a site that is critical for the dimerization of CR2 (Figure 5B). Regardless of the rotamer conformation of the asparagine side chain at this position, the N-linked polysaccharide to this asparagine residue (Figure 5C) would alter dimerization, either by direct or indirect interference with hydrogen bond formation.

An alternative possibility we have considered is whether glycosylation at this site would directly interfere with C3d binding by CR2. However, C3d ligand binding to CR2 occurs via interactions of ligand with the SCR2 domain of CR2 (Szakonyi et al., 2001). Because of the side-to-side packed structure of SCR1-SCR2 demonstrated in the cocrystallized molecules (Szakonyi et al., 2001), the distance between position D53 in SCR1 and the C3d binding interface of SCR2 would, in principle, allow a *cis* inhibition effect on ligand binding by a polysaccharide at the D53 position that has a linear chain length of 10 sugar residues. However, molecular modeling reveals that it would be very unlikely due to steric hindrance for a normally branched N-linked polysaccharide to reach from D53 to the ligand binding interface on SCR2. It is also unlikely that a change in secondary structure of the SCR1 domain due to this polymorphism would have an impact on the ligand binding domain in SCR2. Therefore, it is most likely that the primary functional effects of the SCR1 polymorphism identified in B6.*Slc1c* B cells are mediated by interference with the dimerization interface rather than by direct inhibition of ligand binding.

#### Discussion

We describe here the identification of an altered *Cr2* allele in the *Slc1c* disease susceptibility interval of the

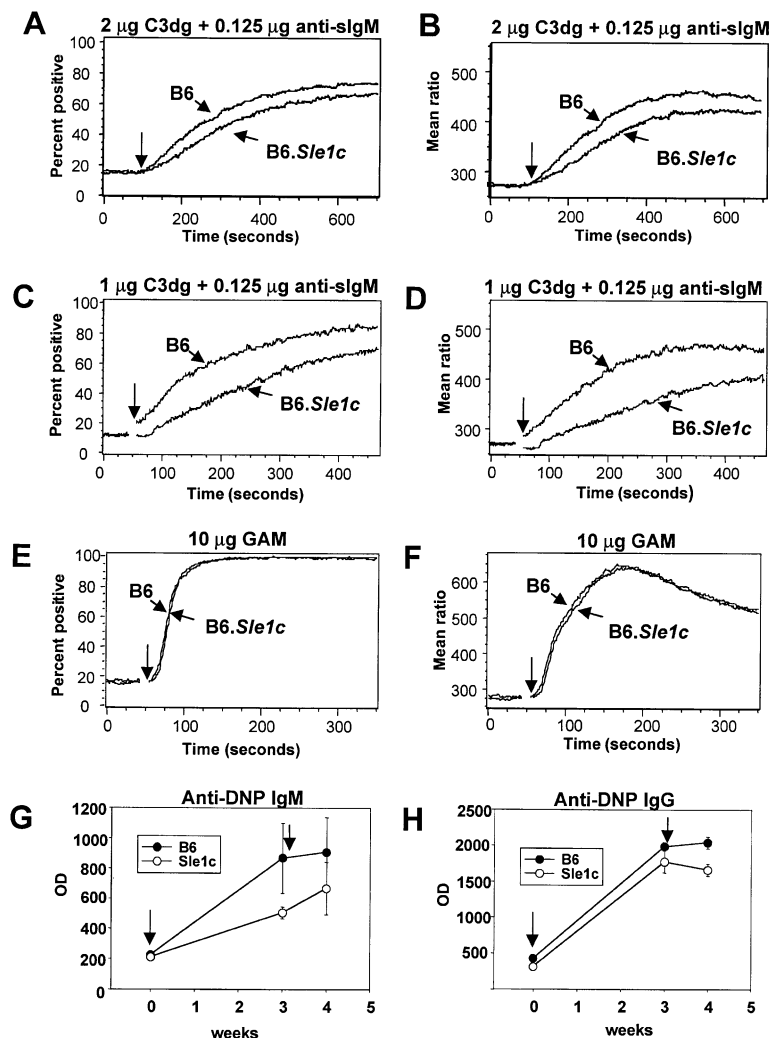


Figure 4. Impaired Functional Responses in B6.Sle1c CR1/CR2

(Panels A–F) Indo1-AM-loaded B cells were stimulated with tetramers prepared with varying amounts of b-rC3dg plus 0.125  $\mu$ g of biotinylated anti-slgM (b-7-6) and 0.2  $\mu$ g streptavidin in order to cocrosslink CR2 and slgM. B220-positive cells were analyzed for increases in the intracellular concentration of calcium. Panels (A) and (B) and panels (C) and (D) represent separate experiments, each using cells purified from a single B6 and a single B6.Sle1c mouse. The percent of cells with intracellular calcium at levels >10% above baseline (A and C) and the mean ratio of bound to unbound Indo1-AM (B and D) were determined. Use of b-rC3dg or biotinylated b-7-6 alone at these doses resulted in no effect on intracellular calcium levels (data not shown). Cells were also activated with a strongly crosslinking anti-slg antibody (F(ab')<sub>2</sub> goat anti-mouse Ig; GAM) with percent cells responding depicted in (E) and mean response in (F). These data are representative of five separate experiments. (Panels G–H) Five 6-week-old B6 and B6.Sle1c mice were immunized intraperitoneally with the T-dependent antigen DNP-KLH in alum on day 0 and on day 21 (arrows). Sera obtained on days 21 and 28 were tested by ELISA for the presence of IgM (G) and IgG (H) anti-DNP antibodies. The mean and SEM of OD readings for 1:100 dilutions of sera are demonstrated.

NZM2410 mouse model for SLE. This allele exhibits multiple polymorphisms when compared to the B6 allele, including a single-nucleotide polymorphism in the ligand binding domain of CR2 that introduces a new N-linked glycosylation site. This site is utilized for glycosylation, as evidenced by the increased molecular weight of CR1 and CR2 encoded by the *Sle1c* allele. Importantly, the presence of a carbohydrate group at this site would interfere with the dimerization of CR2, as predicted by the recently solved crystal structure of SCR1 and SCR2 of human CR2 in complex with the C3d ligand (Szakonyi et al., 2001).

There are several lines of evidence that support our conclusion that *Cr2* is the disease susceptibility gene corresponding to the *Sle1c* locus. First, B6.Sle1c B cells bound rC3dg tetramers at levels much lower than B6 B cells. Since the expression of CR1/CR2 is equivalent on B cells of both strains by flow cytometry and immunoprecipitation of surface-labeled proteins, the decrease in binding is likely due to a decrease in receptor avidity for ligand.

Second, B cells expressing the NZW *Cr2* allele were functionally impaired, in that B6.Sle1c B cells were less able to respond to synergistic signals through CR1/CR2

and slgM. Decreased numbers of B6.Sle1c B cells responded to coligation of CR1/CR2 and slgM, and the amplitude of the response was lower than that in B6 B cells. Furthermore, B6.Sle1c B cells required more time to reach their peak response. The decreased ability of B6.Sle1c B cells to bind ligand may explain why less cells responded, since fewer cells may bind ligand at a level sufficient to activate the cells. The decreased ligand binding capacity of B6.Sle1c CR1/CR2 might also diminish their ability to enhance BCR signaling with comparable kinetics and amplitude to B6.

Third, B6.Sle1c mice were unable to mount normal humoral immune responses to a T-dependent foreign antigen. Immunization with DNP-KLH resulted in a blunted IgG anti-DNP response, similar to, although not as marked as, that seen in *Cr2*<sup>-/-</sup> mice (Ahearn et al., 1996; Croix et al., 1996; Molina et al., 1996; Wu et al., 2000). This was not unexpected, as the *Sle1c* SCR1 polymorphism resulted in a relative decrease in CR1/CR2 function rather than an absolute lack of function, more similar in nature to *Cr2*<sup>+/-</sup> mice which demonstrate an intermediate defect in antibody production (Molina and Holers, 1996; unpublished data).

We have considered how interruption of the CR2 di-



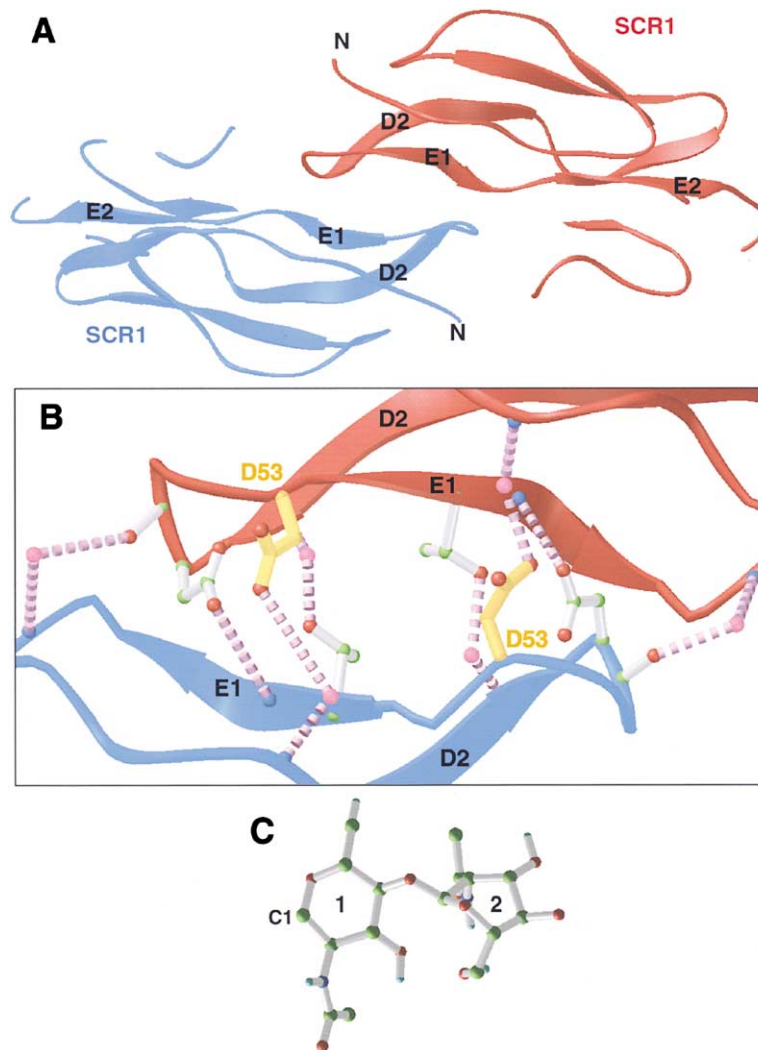


Figure 5. SCR1 Polymorphism in B6.Sle1c CR2 Interferes with a CR2-CR2 Dimer Interface

(A) The overview of the two SCR1 coming together in a CR2 dimer (Szakonyi et al., 2001). Each SCR1 contains four anti-parallel  $\beta$  strands. Strand D2 and E1 of one CR2 molecule (red) pack against strand E1 and D2 of the second CR2 molecule in the dimer (blue). (Prepared using RIBBONS).

(B) Detail of interactions at the SCR1 dimerization interface. The CR2 dimer is formed through hydrogen bonds (dashed line in purple) between side chain and main chain atoms of the SCR1 domain. Some of the hydrogen bonds are formed via water molecules (balls in pink). Residue D53 (corresponding to H52 in mouse CR2) is one of the key residues in the formation of the hydrogen network bonding the two CR2 molecules together. In the SCR1 polymorphism of B6.Sle1c CR2, the D53 position is occupied by an asparagine residue that is glycosylated.

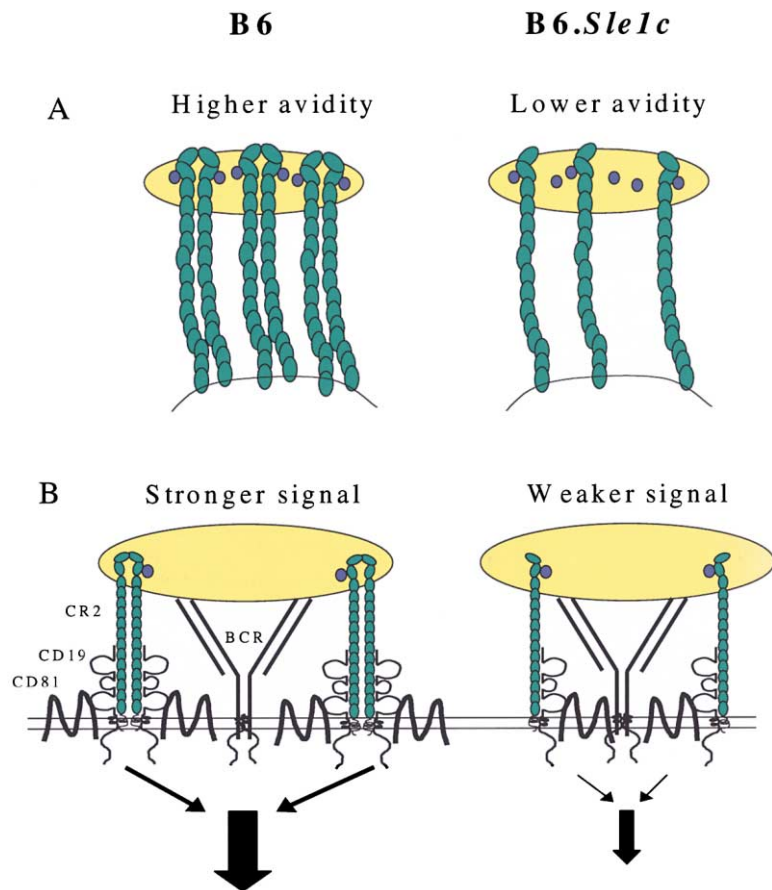
(C) Structure of carbohydrate chain, showing only the first two N-acetyl-glucosamine residues in the N-linked polysaccharide. C1 of the first residue is indicated. Residues are drawn to scale with respect to the SCR1-SCR1 dimerization interface shown in (B). Regardless of the orientation of the glycosylated amino group on the asparagine side chain (due to different rotamer conformations), the presence of even a short carbohydrate chain would interfere with hydrogen bond formation and CR2 dimerization.

merization interface by glycosylation on SCR1 might affect C3d binding, which is mediated through SCR2. Monomeric C3d interacts with CR2 with a very low affinity, whereas polymeric C3d has an affinity that is at least 1000-fold higher (Carel et al., 1990; Moore et al., 1989). In addition, binding of ligand to CR2 appears to have a threshold effect in that significant binding does not occur unless cells express a certain number of receptors, after which binding of ligand is proportional to the level of CR2 expression (Boackle et al., 1997). These data support an important role for receptor aggregation either promoted by or enhancing ligand binding and suggest that a structural alteration that interferes with CR2-CR2 interactions would result in decreased ligand binding. A plausible explanation for these results based on the CR2 crystal structure is that CR2 dimers can be brought together by C3d ligand. CR2 dimerization would increase the local concentration of receptors available for polymeric C3d interactions, thereby increasing the overall avidity of CR2-C3d interactions (Figure 6A). If dimerization is key to promoting high-avidity receptor-ligand interactions, interference with dimer formation would decrease polymeric C3d binding as was seen in our experiments.

Furthermore, interference with the dimerization interface may also result in decreased signals through the BCR, independent of direct effects on ligand binding. Previous studies of C3d acting as a molecular adjuvant have shown that two or three C3d molecules attached to hen egg lysozyme (HEL) can enhance the IgG1 humoral response to HEL by 1000 to 10,000 times (Dempsey et al., 1996). Based on the CR2 crystal structure, we predict that binding of C3d-bound antigen to a single BCR can bring together through CR2 dimers twice as many CR2/CD19/CD81 signaling complexes as a CR2 monomer (Figure 6B), providing a greater enhancement of the immune response.

CR2 dimer interactions have not been detected previously in solution studies using gel filtration or ultracentrifugation (Moore et al., 1989), possibly due to weak interactions, although strong interactions would not be necessary on cell membranes where receptors are largely constrained to lateral movement. Our data suggest that CR2 dimerization plays a physiological role in ligand binding since a polymorphism that would interfere with dimerization results in decreased C3d ligand binding.

It is interesting that NOD and SWR mice share the



**Figure 6. Model of CR2-CR2 Dimer Formation as a Key Component of the CR2 Ligand Binding and Signal Transduction Complex that Is Altered in B6.Sle1c Mice**

(A) Model of CR2 interacting with C3d-bound antigen. The dimer contact is through SCR1 only, as seen in the crystal structure, while C3d contacts SCR2. C3d interactions to CR2-expressing cells in B6 mice are of a higher avidity due to receptor dimerization.

(B) Model of enhanced BCR-mediated signaling induced by C3d-bound antigen crosslinking CR2 dimers and the BCR on the cell surface. The dimer form of CR2, as opposed to the monomer, in complex with CD19/CD81 permits C3d-bound antigen to crosslink additional CR2/CD19/CD81 signaling complexes on the cell surface, augmenting the signal transduced by the BCR.

SCR1 polymorphism with NZW, along with the polymorphisms in SCR7 and SCR10 of CR2 (SCR13 and SCR16 of CR1). These three polymorphisms may mark an allele for Cr2 that confers an autoimmune phenotype when present on the appropriate genetic background. At this time, however, we cannot rule out a role for the other SCR polymorphisms in the effects on ligand binding, although we believe it most likely that the CR2 SCR1 polymorphism is responsible for these effects. It is likely that this polymorphism also explains the decrease in CR2 signaling identified in B6.Sle1c B cells, although the polymorphisms in the transmembrane and cytoplasmic domains of B6.Sle1c CR1/CR2 may contribute to this effect. The transmembrane domain of CR2 is critical for the interaction of CR2 with CD19 (Matsumoto et al., 1993), and the polymorphism in the transmembrane domain of the B6.Sle1c Cr2 allele may interfere with this interaction. However, preliminary experiments have shown that CD19 does coimmunoprecipitate with CR1/CR2 in digitonin lysates of B6.Sle1c B cells (data not shown), suggesting that this signaling complex is at least physically intact. The change from a serine to an arginine in the B6.Sle1c cytoplasmic domain may also impact signaling through CR2, although the functional role of the CR2 cytoplasmic domain has not yet been clearly determined. Our previous studies did show that CR2 lacking the cytoplasmic domain could not be internalized (Carel et al., 1990), providing some evidence for a function of this domain.

Previous studies have suggested that Cr2 deficiency alone on a B6 background does not confer severe autoimmune disease, but may contribute to specific phenotypes (Chen et al., 2000). Indeed, our studies suggest that the expression of the NZW CR1/CR2 allele confers only a modest phenotype (autoreactivity to chromatin). However, we propose that the combination of the NZW CR1/CR2 allele with altered alleles from other disease susceptibility loci can result in a fully penetrant end-stage disease phenotype. We and others have shown that autoimmune diseases result from synergistic interactions between susceptibility alleles that each provide a small contribution to the disease phenotype (Cornall et al., 1998; Morel et al., 2000).

There are several potential mechanisms by which dysfunction of CR1/CR2 might promote the development of autoimmunity. CR1/CR2 may play a role in the development of central tolerance by lowering the threshold for negative selection of autoreactive B cells, as suggested by previous studies using the HEL model (Prodeus et al., 1998). When the expression or function of B cell CR1/CR2 is altered, autoreactive B cells may be able to escape the mechanisms that would normally render them tolerant to autoantigen. These receptors may also be important in the maintenance of peripheral tolerance by continually exposing circulating B cells to autoantigen sequestered in secondary lymphoid organs via interactions with CR1/CR2 on FDC. Finally, CR1/CR2 may play a role in generating autoimmune responses



by their effects on antigen presentation. Binding and internalization of autoantigen by CR1/CR2 may result in the presentation of unique peptides that are recognized by autoreactive T cells (Boackle et al., 1997, 1998). The levels or types of costimulatory molecules upregulated may also vary when CR1/CR2 are coligated with slg (Kozono et al., 1998), resulting in the skewing of T cell cytokine production to create an environment that effects loss of tolerance to autoantigen.

Although the NZW *Cr2* allele generates proteins that are structurally and functionally altered, its role in the pathogenesis of autoimmune disease will not be proven until the *Sle1c* phenotypes are demonstrated to resolve in the presence of normal gene products. This will require the generation of transgenic B6.*Sle1c* mice that overexpress B6 *Cr2* gene products, studies that are in progress. It is possible that incomplete resolution of the autoimmune phenotype will result from these studies, suggesting that other disease-related genes located within the *Sle1c* interval are contributing to the phenotype. We are currently generating recombinant strains to narrow the interval containing *Cr2* so that we can further address this possibility. If our present results are confirmed, *Cr2* will be the first SLE susceptibility gene to be identified by linkage analysis. Additional studies are planned to determine whether a functionally significant polymorphism in CR2 exists in patients with SLE, which would strongly suggest that this gene plays an important role in the pathogenesis of autoimmune disease. Previous studies have already shown that CR2 levels are decreased in patients with SLE (Levy et al., 1992; Marquart et al., 1995; Wilson et al., 1986), supporting either a genetic or acquired mechanism by which alterations in CR2 would influence autoimmunity.

Understanding the functional effects of this altered allele will allow us to better understand its impact on the development and persistence of autoimmune disease. Furthermore, irrespective of its role in autoimmunity, studies of this highly polymorphic allele, which generates structurally and functionally altered proteins, will assist in our understanding of the biology and structure-function relations of CR1/CR2. Both in vivo and in vitro experiments, utilizing knockin strategies of various NZW *Cr2* gene products and expression of various recombinant NZW CR1/CR2 proteins, will be utilized to clarify the function of these receptors and their domains in normal and autoimmune responses.

## Experimental Procedures

### Mice

The production of the B6.*Sle1c* subcongenic strain from B6.*Sle1* has been previously described (Morel et al., 2001). Mice were bred and maintained under identical conditions at the University of Colorado Health Sciences Center for Laboratory Animal Care or the University of Florida Department of Animal Resources. C57BL/6 (B6) mice were obtained from the National Cancer Institute (Bethesda, Maryland) and the Jackson Laboratory (Bar Harbor, Maine).

### Immunoprecipitation and SDS-PAGE Analysis

Splenic cell suspensions were depleted of RBC with Gey's solution and surface biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, Illinois). Cells were lysed with RIPA buffer supplemented with Complete protease inhibitor cocktail tablets (Boehringer Mann-

heim, Indianapolis, Indiana), 10  $\mu$ g/ml pepstatin, and phosphatase inhibitors (0.05 mM sodium orthovanadate, 50 mM sodium fluoride, and 20 mM  $\beta$ -glycerophosphate). Lysates were incubated with rat anti-mouse CR1/CR2 (7E9), rabbit anti-mouse CD19 (rabbit anti-serum, produced by S.A.B.), or rat anti-mouse CD22 (Southern Biotechnology Associates, Birmingham, Alabama) for 30 min on ice, followed by addition of Protein G Sepharose (Amersham Pharmacia, Piscataway, New Jersey) and incubation for 1 hr at 4°C. Nonreduced samples were analyzed by 8% SDS-PAGE, transferred to a nitrocellulose filter, and incubated with HRP-streptavidin (Zymed, San Francisco, California). In some experiments, immunoprecipitated proteins were deglycosylated with 0.6 U PNGaseF (New England Biolabs, Beverly, Massachusetts) for 2 hr at 37°C under nondenaturing conditions prior to SDS-PAGE analysis.

### Sequencing of *Cr2*

RNA was extracted from spleens of each of the following strains: B6.*Sle1c*, B6, NZW, NZB, BALB/c, SWR, NOD, and MRL using Trizol reagent (Life Sciences Technologies, Grand Island, New York). cDNA was generated by reverse transcription using random hexamers (Applied Biosystems, Foster City, California), and PCR products generated from cDNA using the following primer sets:

Cr2-1 - 5'-CTCTTCTCTCTCTGCTACAGGC-3'  
Cr2-2 - 5'-GGAGATGGCTGAGGTGACTC-3'  
Cr2-3 - 5'-CATTGGTGACTCGTCTGCTACATG-3'  
Cr2-4 - 5'-GAGGTGGTAGACATCCCATGAAGC-3'  
Cr2-5 - 5'-CCATGAAAGGCAGCAGAATAGCATG-3'  
Cr2-6 - 5'-GACTTCAGGAGGAGGGTCAAG-3'  
Cr2-7 - 5'-GCAAACCTTCTAGTCAGTCTATCCCAG-3'  
Cr2-8 - 5'-GTCACAGACAACCAGCAACAAAC-3'  
Cr2-9 - 5'-CCCTCTGAGTGCCCATCAC-3'  
Cr2-10 - 5'-GTGAAGCTCAGCCTTCTCTG-3'  
Cr2-11 - 5'-CTACACCTGTGACCAAGCCC-3'  
Cr2-12 - 5'-CCTACTGGCTTACACTCTGCAACTG-3'  
Cr2-13 - 5'-GCTGTGACCCTGGCTATTACTGG-3'  
Cr2-14 - 5'-CGGATCTGACTGCTTCCACTC-3'  
Cr2-15 - 5'-GTCCAGTGACACAGACGTTTCATG-3'  
Cr2-16 - 5'-GTCCACTCCAAGGCCATGACC-3'  
Cr2-17 - 5'-GGTCATGGCTCTTGGAGTGGAC-3'  
Cr2-18 - 5'-GATAAGACGTGCCTCTCCAGCC-3'  
Cr2-19 - 5'-GGCTGGAGAGGCACGTCTTATC-3'  
Cr2-20 - 5'-GGTGGCATGTAAGCAGATATGGTG-3'

PCR, performed in a GeneAmp PCR System 9700 (Applied Biosystems), generally utilized touchdown PCR cycling parameters (94°C  $\times$  2 min, 25 cycles of 94°C  $\times$  30 s, 65°C  $\rightarrow$  55°C  $\times$  30 s, 72°C  $\times$  1 min, followed by 15 cycles of 94°C  $\times$  30 s, 55°C  $\times$  30 s, 72°C  $\times$  1 min, then 72°C  $\times$  10 min). PCR products were purified with the QIAquick PCR Product Purification Kit (QIAGEN, Santa Clarita, California) and sequenced with the above primers by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility using ABI Prism kits (Applied Biosystems) containing AmpliTaq DNA Polymerase FS in dRhodamine Terminator Cycle Sequencing Ready Reaction kit (part number 404043). The reaction products were analyzed on either an ABI 373A or an ABI Prism 377 DNA fluorescent sequencer (both standard or XL versions) (Applied Biosystems). Polymorphisms resulting in the loss or gain of restriction site were screened on a panel of eleven mouse strains by RFLP-PCR with DNAs purchased from the Jackson Laboratory.

### Flow Cytometry

Splenic cell suspensions were depleted of RBC with Gey's solution and Fc $\gamma$ RII receptors blocked with 2.4G2 (American Type Tissue Collection, Rockville, Maryland). B cells were incubated with saturating amounts of biotinylated rat anti-mouse CR1 (8C12) or rat anti-mouse CR1/CR2 (4E3), followed by PE-streptavidin (Southern Biotechnology Associates) and FITC-B220 (Pharmingen, San Diego, California). In separate experiments, rC3dg tetramers were prepared by incubating 2  $\mu$ g of in vivo biotinylated recombinant human C3dg (Henson et al., 2001) with 0.4  $\mu$ g PE-streptavidin in a total volume of 100  $\mu$ l for 30 min at room temperature. 100  $\mu$ l of PE-labeled C3dg tetramers in combination with FITC-B220 were incubated with  $1 \times 10^6$  cells for 30 min at 4°C. Cells were analyzed on a FACSCalibur

(Becton Dickinson, San Jose, California) and mean amount of fluorescence bound was determined.

#### Intracellular Calcium Measurements

Splenic cell suspensions were depleted of RBC with Gey's solution. Cells were loaded with Indo-1AM (Molecular Probes, Eugene, Oregon) and B cells labeled with FITC-B220. Sufficiency of Indo-1 loading was assessed by stimulation with 10  $\mu$ g of F(ab')<sub>2</sub> polyclonal goat anti-mouse slg (Southern Biotechnology Associates). Tetramers prepared by incubating various amounts of b-rC3dg and/or biotinylated rat anti-mouse IgM (b-7-6, provided by Dr. John Cambier) with 0.2  $\mu$ g streptavidin (Biosource, Camarillo, California) were added to  $2 \times 10^6$  cells and calcium flux of B220<sup>+</sup> cells was measured on a BD LSR (Becton Dickinson, San Jose, California). Results were analyzed using FlowJo software (Tree Star, Inc., San Carlos, California).

#### Immunization and Analyses of Humoral Immune Responses

Five 6-week-old female B6.Sle1c and B6 mice were immunized intraperitoneally with 10  $\mu$ g DNP-KLH in alum (1:1) on days 0 and 21, then sacrificed on day 28, at which time serum titers to DNP were determined by ELISA. ELISAs were performed by coating microtiter wells with DNP-BSA, followed by sequential incubations with sera, HRP-conjugated anti-isotype antibodies, and substrate, as previously described (Mohan et al., 1997). The mean OD at 405 nm for each mouse was calculated from duplicate wells.

#### Molecular Modeling

The human CR2 dimer from the CR2-C3d complex structure (Szakonyi et al., 2001) was used for modeling mouse CR2 using the program "O" (Jones et al., 1991) on a Silicon Graphics workstation. Oligosaccharide sugar residues used in the modeling were downloaded from the PDB database [1ZXQ, (Casasnovas et al., 1997)].

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